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The Effects of SNMP-2 Gene Expression on Mating Discrimination in Male Drosophila Melanogaster

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The effects of SNMP-2 gene expression on mating discrimination in male *Drosophila melanogaster*

by

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Dedication

For all of the maggots.

Acknowledgements

Thank you to Dr. Vogt for taking me into his lab despite knowing how little experience I had. I had expressed an interest in Neuroscience and harassed him until he finally let me into his class. I appreciate the time and effort put into teaching me fundamentals of working at the bench; the extent of my inexperience being that I could not even manage a micropipette. This was a great experience for me, strange and hilarious as it was. I learned patience, perseverance, and a lot about flies.

Thank you to Richard Fandino for explaining the basics and teaching me enough to understand what it was I was attempting to accomplish. I am also thankful for the afternoon brews and humorous exchanges.

Thank you to my family for the ruthless mockery and financial support.

Abstract

In order to distinguish between relevant and extraneous stimuli, insects have adapted specialized processes to perceive cues that are beneficial for survival and proliferation. Volatile molecules in the environment can stimulate olfactory receptors (ORs) and gustatory receptors (GRs) in chemosensory organs called sensilla. Specialized proteins located within these sensilla guide and assist chemosensory molecules to the receptors, which then trigger a transduction pathway that elicits behavioral responses. Sensory Neuron Membrane Proteins (SNMPs) are transmembrane proteins found on both gustatory and olfactory sensory organs in insects. There are two forms of these proteins, SNMP-1 and SNMP-2. In *Drosophila melanogaster,* the function of the SNMPs is currently unknown, but it is thought to contribute to proper recognition of pheromones secreted by male *Drosophila.* To determine the role of SNMP-2 in *Drosophila,* we reduced the gene expression of SNMP-2 by targeting the gene with RNA-mediated gene interface (RNAi). We then recorded courtship displays of male-male and male-female interactions and found that reduction of SNMP-2 increased the frequency at which males courted other males, but did not affect that of which males courted females. Results were confirmed by quantitative real time PCR.

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Chapter 1

Introduction

1.1 Insect Olfaction

Every animal is presented with a myriad of choices on a day-to-day basis. What to eat, where to live, who to avoid, and with whom to reproduce are all decisions that must be made as a part of life. As human beings, we employ a slightly more sophisticated process than other animals when it comes to selections. Whereas humans and other more evolved species rely on conscious thought and emotional stimulation that factor into decision-making, insects essentially simply react to external stimuli in an endogenous manner of reflex responses. One primary example is the instance of courtship. The human race has established a plethora of dating websites and social events to facilitate the process of filtering through diverse prospects in order to find a compatible mate. Insects release and detect volatile chemical compounds, or pheromones, to signify the availability for courtship and mating. In this regard, as well as with finding food and circumventing predation, insects' interaction with the environment is more of a reaction to chemical cues than it is a deliberate decision.

The chemosensory system is a very complex network of neurons and receptors that allow the insect to respond appropriately to external stimuli. To distinguish between relevant and extraneous stimuli, insects have adapted specialized processes to perceive cues beneficial for survival and proliferation. In the case of *Drosophila melanogaster*,

chemosensory organs are located on the legs, wings, head, and thorax. Volatile molecules in the environment can stimulate olfactory receptors (ORs) and gustatory receptors (GRs) in these chemosensory organs, triggering a transduction pathway that elicits a behavioral response. One example of the efficacy of this complex chemical communication is the ability of an adult male to determine whether or not a female has previously mated merely by coming into contact with her, as certain pheromones are transferred from male to female during courtship and can be detected by the specific receptor OR67d (Ziegler et al., 2013).

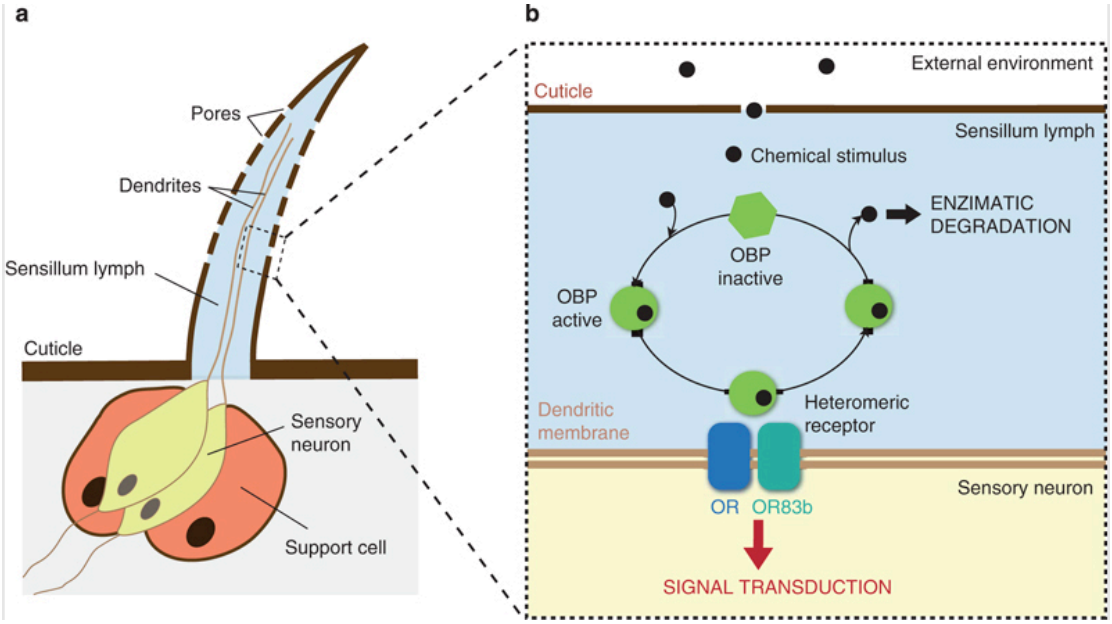

Figure 1.1

(A) Diagram of chemosensory sensilla including cuticle, olfactory dendrites, pores, sensillum lymph fluid, sensory neurons, and support cells. (B) Schematic of interaction of chemical stimulus entering sensillum lymph, binding with odorant binding protein (OBP), and stimulating heteromic odor receptor bound to the dendritic membrane of a sensory neuron which triggers signal transduction. Chemical stimulus is then degraded by odorant degrading enzyme (ODE, not shown). (Fron Sanchez-Gracia A, Vieira F, Rozas J. 2009.)

Chemosensory organs used to detect volatiles, including pheromones, are called sensilla; they are hair-like projections providing a barrier between the environment and the chemosensory neurons transmitting signals that are subsequently perceived as taste and smell. A chemosensory molecule enters the hollow, lymph-filled sinus of this organ through one of many pores in the cuticle wall at the tip of the projection. The molecule then dissolves in the lymph, which bathes the dendrites of one to five chemosensory neurons within the lumen, and activates an olfactory receptor protein (OR) or a gustatory receptor protein (GR). Olfactory receptors are expressed with the co-receptor ORCO, which acts as an ion channel and contributes to signal transduction (Vosshall & Stocker, 20007). Other proteins involved in chemosensory perception are Odorant Binding Proteins (OBPs), soluble proteins that bind chemosensory molecules and deliver them to odor receptors, Odor Degrading Enzymes (ODEs) that remove volatiles within the lumen, and Sensory Neuron Membrane Proteins (SNMPs) that have not yet been completely characterized. Sensilla surrounding gustatory neurons are called taste bristles (TBs), have a single terminal pore and contain mechanosensory neurons. The sensilla surrounding olfactory neurons differ in that they have multiple pores in the cuticle, no mechanosensory neuron, and include OBPs and ODEs that contribute to odor detection (Galindo and Smith, 2001).

There are three types of olfactory sensilla: basiconic, coeloconic, and trichoid. Of these three types, only the trichoid sensilla are required for pheromone recognition and social interactions, as proven by their sensitivity to the *Drosophila* pheromone 11-*cis*-vaccenyl acetate (cVA) (Ha and Smith, 2006). Trichoid sensilla are single walled projections containing no pores at all or containing numerous pores that are only 10 nm

in diameter. Extending into the lengthy spine-shaped shaft are one to three unbranched dendrites of olfactory sensory neurons (OSNs) (Stocker 1994). Basiconic sensilla are innervated by OSNs containing odor receptors that respond to food odors (Couto et al., 2005; reviewed Vosshall and Stocker, 2007). They are covered with one cuticular wall containing multiple pores about 30nm in diameter. There are two subtypes of basiconic sensilla, large and small. The large basiconic sensilla can hold four to five neurons whereas the small basiconic sensilla only contain two. Studies done by Stocker and Gendre (1989) indicate that basiconic sensilla do not contain olfactory neurons that detect pheromones pertinent to mating virgin females (Stocker 1994). Coeloconic sensilla enclose OSNs that express different types of ORs, ionotropic receptors (IRs), and are involved in detection of ammonia, carboxylic acid, and water (Yoa et al., 2005). These are double walled cone-shaped sensilla with around ten vertical grooves in the cuticle, covering the lymph that contains dendrites of three OSNs. It is thought that all three of these sensilla house neurons that contribute to olfactory perception; however, supporting physiological evidence is indeterminate (Stocker 1994).

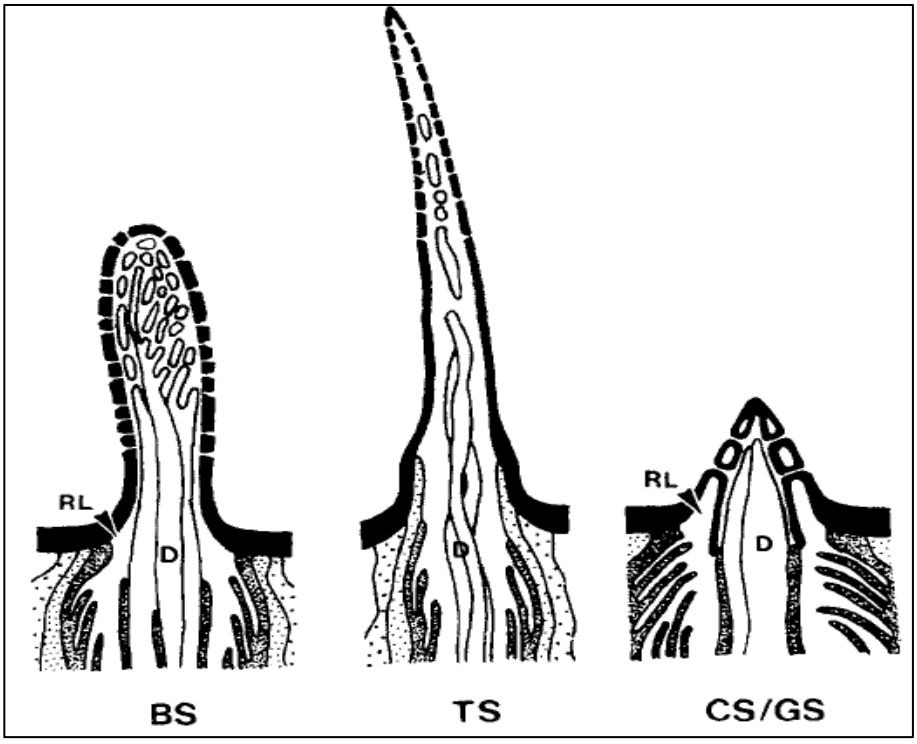

Figure 1.2

Structure of Olfactory Sensilla (adapted from Stocker 1994). From left, BS represents basiconic sensilla, TS trichoid sensilla, and CS coeloconic sensilla. D labels the dendrites housed within the cuticular wall and RL denotes outer receptor lymph space.

Sensilla containing neurons stimulated by olfactory cues are primarily located in the third antennal segment in *Drosophila* (Yao et al 2005). Usually, each dendrite of OSN that is contained within these sensilla expresses only one type of OR specific to a certain range of volatiles. All of the axons of OSNs in a particular region containing the same OR will join to form a glomerulus in the CNS, specifically in the antennal lobe of the brain. These glomeruli exchange signals via local interneurons, which are primarily inhibitory, before stimulating projection neurons that carry messages onto higher level processing (Martin et al. 2011). Gustatory sensilla are widespread throughout the body, developing on the wings, legs, labellum, pharynx, and on the genetalia. These sensilla

contain up to five gustatory neurons that express gustatory receptors and transmit signals to the thoracic ganglion or to the subesophageal ganglion in the CNS.

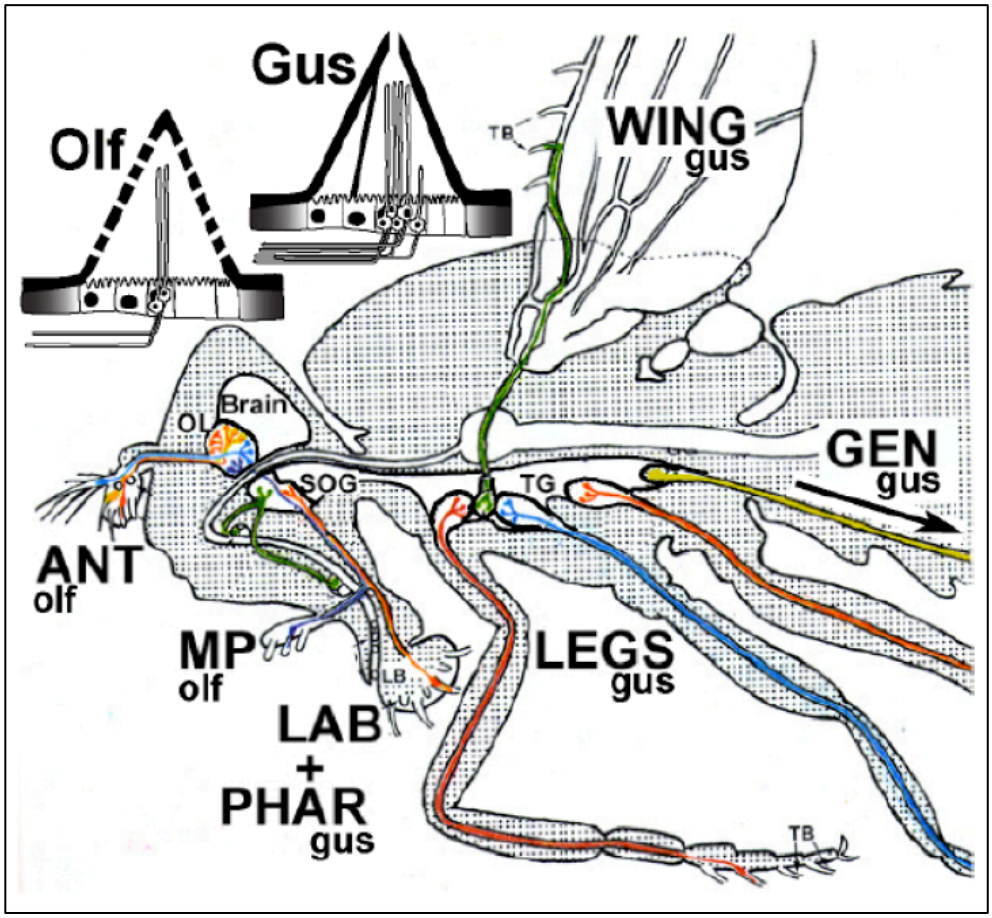

Figure 1.3

Diagram of Adult *Drosophila melanogaster* chemosensory neurons (adapted from Stocker 1994). Olfactory sensory neurons are primarily located in the antennae (ANT) and the maxillary palps (MP); these neurons project to the olfactory lobe (OL) in the brain. Gustatory Neurons send signals from the labellum (LAB), pharynx (PHAR), legs, wings, and genetalia (GEN) to the thoracic ganglion (TG) or the subesophageal ganglion (SOG) in the CNS.

1.2 Sensory Neuron Membrane Proteins

Sensory neuron membrane proteins (SNMPs) are transmembrane proteins found on both gustatory and olfactory sensory organs in insects. SNMPs belong to a larger family of proteins characterized by the human fatty acid transporter (FAT) CD36, a class B scavenger receptor important in recognition and transport of lipids (Benton et al. 2007). This membrane bound receptor has been proven to implement various functions including, but not limited to, cholesterol transport and cell-cell recognition in taste receptor cells. Whereas the insect CD36 homologs, epithelial membrane protein (emp), Croquemort, Peste, NinaD, and Santa maria, are essential in cytoadhesion, carotenoid transport, and chemoreception; the SNMPs' function has not been completely characterized (Nichols and Vogt, 2007). There are four hypothetical models of the functionality of the membrane bound SNMP in Olfactory Sensillum. SNMP could function as a protein receptor, as a protein involved in unloading chemosensory molecules, as a complex with a receptor, or as a protein used as an internalizing mechanism.

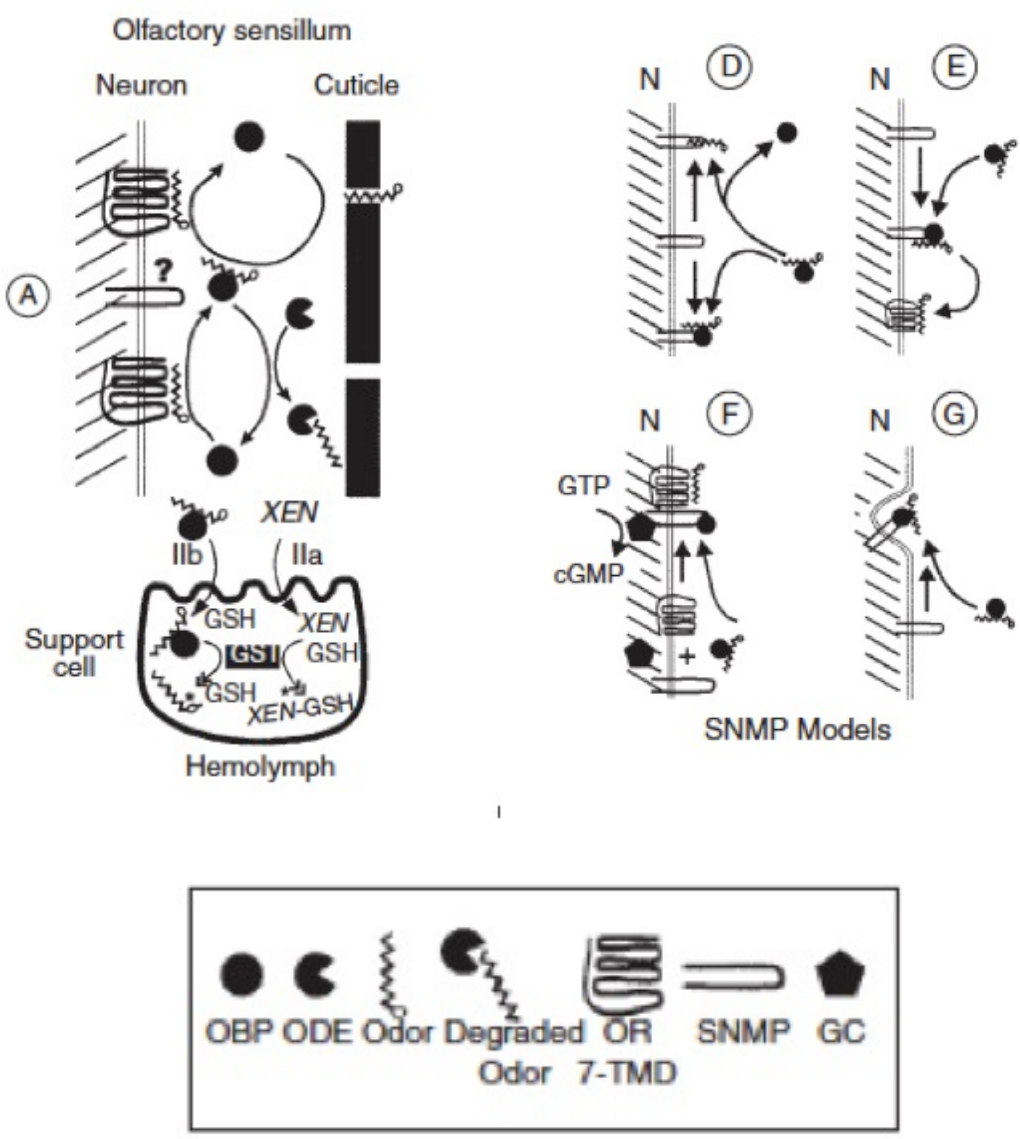

Figure 1.4

(A) Diagram of model of membrane bound SNMP and its interaction with odors, odorant binding proteins (OBPs), odor degrading enzymes (ODEs), degraded odors, Odor Receptor 7-TMD (OR 7-TMD), and guanylate cyclase (GC). SNMP as a receptor (D), as an unloading protein (E), as a complex with a receptor (F), and as an internalizing protein (G).

In insects, these proteins are localized in olfactory sensory neurons of chemosensory sensilla; however, the different subtypes, SNMP-1 and SNMP-2, are unique in their expression patterns. In promoter driven GFP expression, both SNMPs

associate with chemosensory organs throughout the adult body of *Drosophila melanogaster* in the sensilla covering the maxillary palps, labellum, wings, and legs; but the two proteins express in distinct cells.

SNMP-1 is known to express in olfactory neurons of trichoid sensilla and has been classified as pheromone specific; it is essential in the detection of *cis*-vaccenyl acetate (CVA), a volatile organic compound in *Drosophila* that contributes to mate recognition and aggregation behavior (Benton et al., 2007). CVA detection is accomplished by the collaboration of the odorant receptor Or67d, the extracellular pheromone-binding protein LUSH, and SNMP-1 (Jin et al., 2008). Although SNMP-1 and SNMP-2 typically express in the same sensilla, they are never found within the same cell.

SNMP-2 expression is seen in OSNs of coeloconic sensilla, in gustatory neurons found in TBs, and in some support cells associated with olfactory sensilla. It has been shown that a genomic deletion of SNMP-2 in male *Drosophila* leads to a substantial increase in courtship and mating behavior towards other males. This deletion was generated through ends out homologous recombination, a targeted excision resulting in the SNMP-2 knockout (Sparks, PhD Dissertation 2012). In a behavioral comparison between wild type flies and the aforementioned knockout, there was a significant statistical difference, indicating that SNMP-2 could contribute to proper gender recognition during courtship.

Expected response from male-female courtship is a high percentage of time spent in courtship, and the expected response from male-male courtship is a very low percentage of time spent in courtship (discussed in detail in section 1.3). In earlier studies

designed to characterize the role of SNMP-1, the gene was knocked out and showed a resulting phenotypic abnormality. Male-female courtship returned a decreased response, whereas male-male courtship was unaffected. When rescuing the SNMP-1 knockout, male-female courtship behavior returned to normal with the reinstatement of SNMP-1 production. We know that SNMP-1 is required for the proper recognition of CVA (Benton et al, 2007), and it can be concluded from this study that the role of SNMP-1 is directly related to only male-female courtship (Sparks, 2012).

When viewing the effects of SNMP-2 knockout on behavior, it was evident that the frequency of male-female courtship was unaffected while the frequency of male-male courtship was increased. An attempt was made to rescue the gene in order to confirm that the behavioral inadequacies were exclusively due to SNMP-2 gene deletion, yet the effort was unsuccessful. Since the attempt to rescue the knockout failed, another approach to confirm the effect of an SNMP-2 deficiency is necessary. If the absence of the gene, rather than residual effects of the genetic excision, is the origin of the behavioral aberration, it can be assumed that an independent method of decreasing gene expression will have the same phenotypic effect.

Table 1.1
Table illustrating the behavioral effects of knocking out SNMP-1 and SNMP-2 proteins with regards to male-female and male-male courtship.

Genotype	Male-Female courtship	Male-Male courtship
SNMP-1 knockout	Decreased (low)	Normal (none)
SNMP-2 knockout	Normal (high)	Increased (high)

1.3 Mating Behaviors

Normal mating behavior has been characterized as a sequence of courtship behaviors that a male exhibits towards a female (Figure 1.4). The male first orients himself towards a female, then taps her, sings to her by vibrating or flicking one wing, licks the female's genitalia, and finally curls his abdomen in an attempt to copulate with her. Males show more affinity towards females who have not recently mated due to the ability of receptor OR67d to detect compounds secreted by females after copulation. At any time during courtship, the female may refuse copulation or accept the advances of the male by reducing her activity and opening her genitalia (Ziegler et al., 2013). The progression of courtship behavior is species specific and genetically determined, as previously proven by the observation of several mutants' inconsistency with typical courtship displays (Sokolowski, 2001).

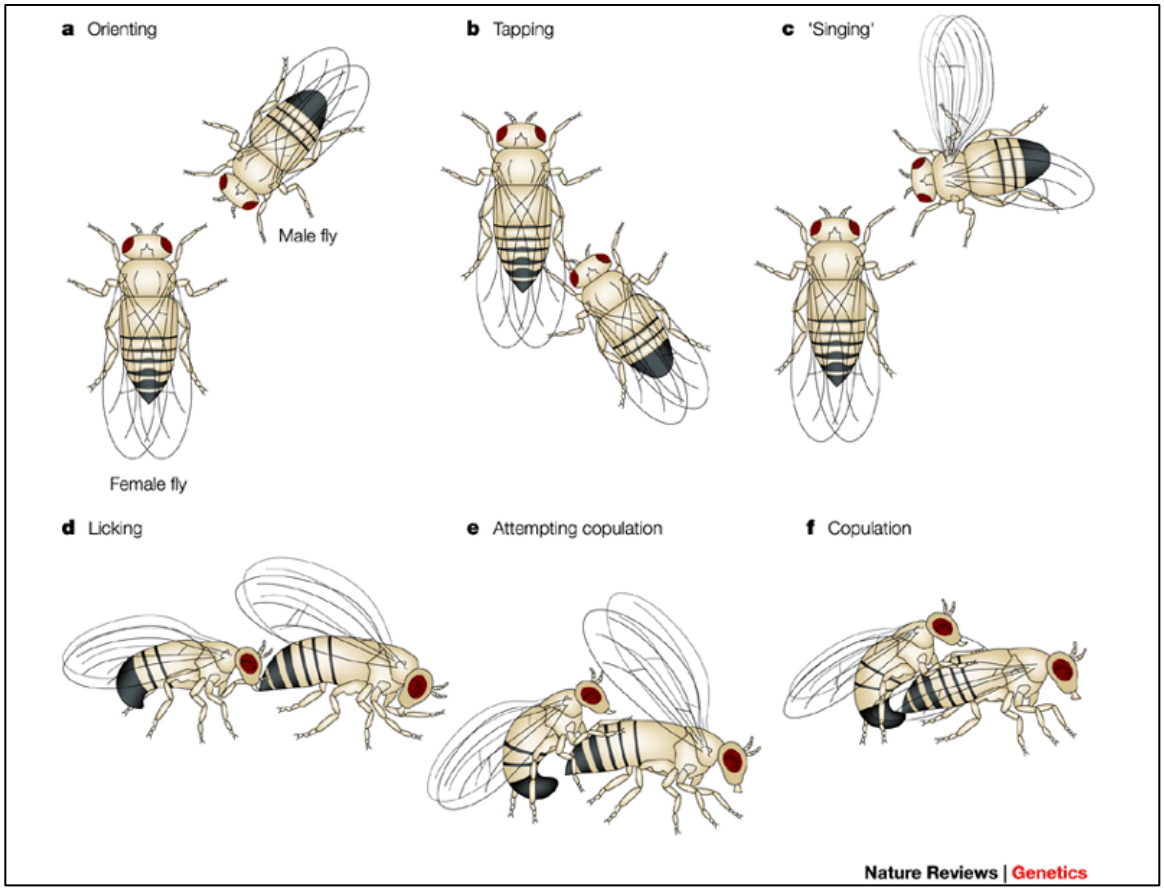

Figure 1.5

(From *Drosophila:* Genetics meets behavior. Sokolowski, M. 2001). Progression of male courtship behaviors. A. The male orients himself towards the female. B. He taps the female with his front legs. C. He vibrates his wings to 'sing' to the female. D. The male will lick the female genetalia to taste for chemical compounds. E. Male will attempt copulation with the female. F. Copulation.

1.4 RNA interference

The conventional sequence in the SNMP-2 KO male is undisturbed; therefore we know that the SNMP-2 gene is not involved in the characteristic courtship behavior. However, previously collected data suggests that SNMP-2 KO males demonstrate a greater propensity than that of W1118 males to engage in courtship behavior with other males (Sparks). This research may suggest that SNMP-2 plays a role in inhibition of

male/male courtship or in accurate gender recognition. As a means to support our belief that SNMP-2 does indeed contribute to normal mating behavior, we will employ another manner of reducing expression of SNMP-2 protein and record any variations seen from standard mating displays.

It may be possible to reduce the gene expression of SNMP-2 by targeting the gene with RNA-mediated gene interface (RNAi). In the endogenous method, RNA molecules bind to and destroy certain mRNA molecules, which consequently prevents gene expression. This phenomenon can be induced in *Drosophila* by crossing a transgenic strain containing UAS-RNAi construct with another transgenic strain containing the GAL-4 transcription factor. UAS-RNAi flies were obtained from the Vienna Drosophila RNAi Center; the particular transgenic line used is of the GD library, created by p-element insertion into wild-type (W1118) flies. GAL-4 binds to the UAS promoter and drives the expression of double stranded hairpin RNAs, which are cleaved into siRNAs by the enzyme Dicer. The siRNAs then recognize specific sequences of the animal's mRNA and degrade it, preventing translation into protein (VRDC 2013, Clemens et al, 2000). Since the flies will contain SNMP-2:GAL4 and UAS:RNAi, only mRNA destined to be translated into the SNMP-2 protein will be degraded. If expression of SNMP-2 is, in fact, reduced, and there is an obvious inconsistency in male courtship behavior as observed with the SNMP-2 knockout, then it can be assumed that SNMP-2 is required for proper gender recognition.

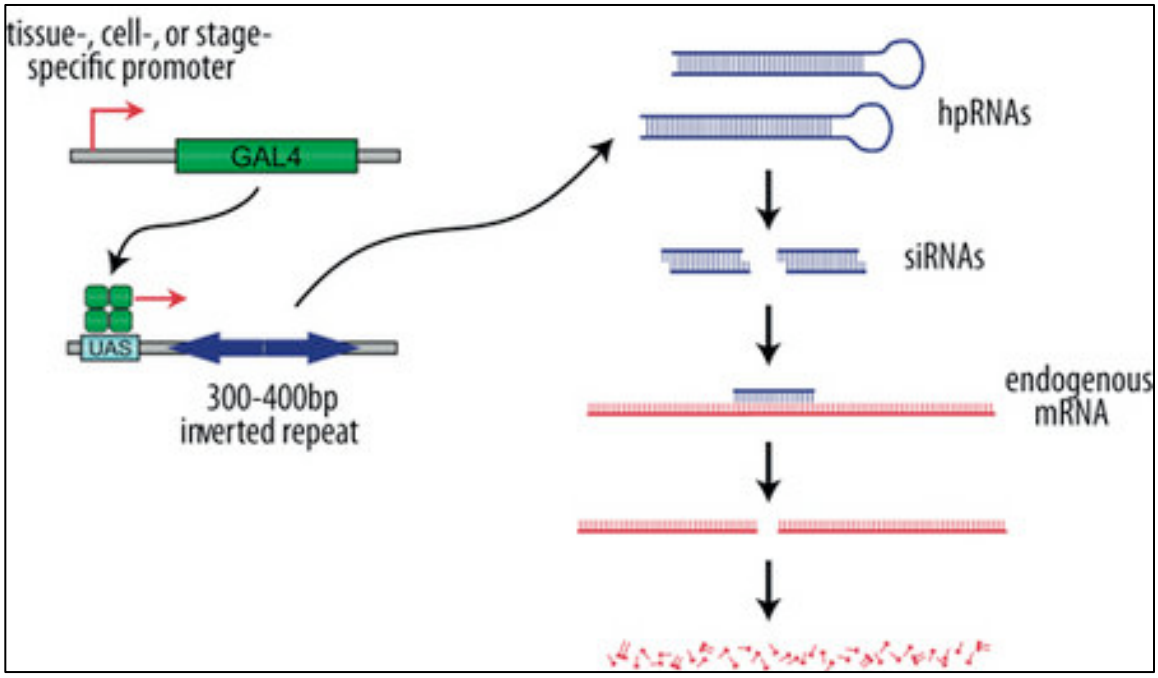

Figure 1.6

(From Vienna *Drosophila* RNAi Center) Illustration of the means by which RNAi targets specific mRNA.

Chapter 2

Methods

2.1 Drosophila Genetic Crosses

Flies were raised at room temperature on a light cycle of 16h day to 8 h night. They were raised in tubes containing a standard mixture of cornmeal, molasses, water, agar, and the anti-fungal agent tegosept.

Previously, a transgenic fly was constructed with the SNMP-2 upstream region driving GAL4 (Sparks, 2012) inserted into a w^{1118} line, wild type except for mutant white eyes. Flies containing the p-element transformation recovered the red eye gene; these were crossed with a fly marked by the phenotypic marker stubble, a genetic insertion on the third chromosome that marked the adults with stubbly hair on the thorax.

For this particular experiment, animals were collected and sorted as pupae; therefore, a different phenotypic marker was needed. To accomplish this, virgin female [SNMP2-Gal4/Tm3,Sb] flies were crossed with another third chromosome marker, [Tm6,tb/Tm6,tb], to balance the SNMP2-Gal4 insertion over a tubby marker. The SNMP2-Gal4 insert was now balanced by the tubby marker, which showed a phenotypic representation of a short, slightly fatter pupal formation. In addition to physically marking the SNMP-2 genetic insertion, the tubby balancer prevented this homozygous lethal gene from genetic recombination.

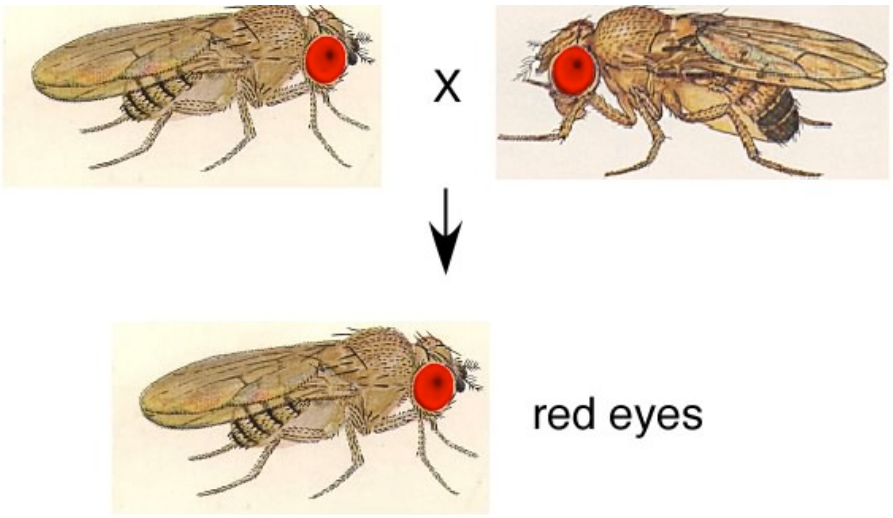

Figure 2.1

Cross [SNMP-2:Gal4/Tm6,Tb] virgin female with [UAS:RNAi/UAS:RNAi] male; red eye marks p-element insertion. Collect F1 red- eyed progeny of cross that lacks tb balancer- will have [UAS:RNAi/SNMP-2:Gal4] system which drives RNAi.

Once the new line had stabilized, this fly was crossed with another (obtained from the Vienna Drosophila RNAi Center) containing a UAS: RNAi insertion, homozygous for this allele, intended to target and silence the SNMP2 gene. Progeny containing both constructs, the SNMP-2:GAL4 and UAS:RNAi alleles, were selected for by recognition of retention of red eye and loss of tubby phenotypes. This combination of phenotypic markers should reflect inclusion of the two genes that should theoretically exhibit inhibition of SNMP-2 translation. When the progeny of this cross began to pupate, non-tubby pupae were collected for behavioral assays, as these were the progeny carrying both SNMP-2:Gal4 and UAS:RNAi. If the gene silencing was successful and relevant, we predicted that the resulting behavior would demonstrate an increase in courtship with male flies due to the absence of olfactory cues in mating behavior. As a control, adult male [W1118] and adult male [+/+; UAS:RNAi/UAS:RNAi; +/+] were placed in the same environment and observed.

2.2 Behavioral Assays

The four strains of *Drosophila melanogaster* used for the behavioral assay are as follows: Canton S, W1118, UAS:RNAi/UAS:RNAi, and SNMP-2:GAL4/UAS:RNAi. Once the larvae had pupated and begun to pigment, an indication of nearing complete metamorphosis into adulthood, they were collected and isolated into 2ml centrifuge tubes (Fisher brand Snap-Cap Flat-Top Graduated, cat# 02681258) with a small amount of food in the bottom and a small hole in the cap (flame heated syringe needle). Isolated pupae were kept in an incubator at 25° C with lights on at 11:00, off at 3:00 hours. Eclosure was noted daily (~18:00 hours) as adults emerged from the pupal case in isolation of the 2ml centrifuge tubes. Isolation was necessary to ensure social naiveté prior to the observed courtship trials. Following eclosure, the adults remained in the incubator for 3 to 5 days to mature, as this is the window of the reproductive peak in adults. Behavioral assays were conducted within the first 4 hours of light, the time at which flies are most active.

A watchglass 40 mm in diameter (Sigma-Aldrich, Z509205-1PAK) and rectangular (2X3") glass plate (1/8" window glass) underneath formed the chamber that enclosed the space within which the animals would interact. Target animals, males and females of the strain Canton S, were anesthetized with ice, placed onto a chilled petri dish and decapitated with a #11 scalpel blade. Headless target animals and males to be tested were blown into a small hole in the glass plate through flexible plastic tube with a blue pipette-tip glued to the end. Test animals were introduced to the chamber first in order to acclimate, followed by their headless targets after approximately five minutes. Up to six pairs of flies were recorded simultaneously over a period of ten minutes with a Kodak

PlaySport (Zx5) HD Waterproof Pocket Video Camera. The assays were video recorded in black and white, illuminated only by a far-red LED at 650-670nm, a wavelength not visible to the animals as visual acuity could potentially affect mating selectivity.

Figure 2.2

Black and White photograph of behavioral assays showing two flies engaged in courtship behavior.

2.3 Statistical analysis on behavioral assays

Once the courtship data had been collected, the times that the animals spend completing recognized sequences of courtship behavior in the videos were observed and recorded. The number of pairs in courtship and number and percentage of non-responding animals were calculated as well as the mean, median, and standard variation

of the percentage of time spent in courtship. This quantitative data was analyzed using a non-parametric technique, as the numbers recorded are a representation of preference. Statistical analysis to determine p-value was performed using the Mann-Whitney test (Zhou, C et al. 2012). Whisker plot was created to visually represent the non-parametric data. Whiskers indicate the farthest data points no farther than 1.5 times the interquartile range (50% of the data around the median), outlined by the box. Horizontal line within the box indicates the median of the values.

2.4 Quantitative PCR

Extent of the RNAi efficiency was confirmed through quantitative real-time PCR after Bohbot and Vogt (2002). Primers were designed from published cDNA sequences and were used to amplify DNA from the [SNMP-2/UAS:RNAi] tissue. Amplification of mRNA transcripts was recorded in real-time.

Table 2.1

Primers used for qPCR amplification and PCR for each protein. Sense followed by antisense. SNMP-1 primers to amplify SNMP-1 mRNA, SNMP-2 primers to amplify SNMP-2 mRNA, DmRP49 primers to amplify ribosomal mRNA as a control.

SNMP-1 sense	GAGGAACACGTTCATTTTCAACC
SNMP-1 antisense	TTAATCCTTTGGAAACCAGCTCC
SNMP-2 sense	TGCACATGAATGCATTTTTACAAG
SNMP-2 antisense	GCAGCACAGATTTACGTTTCC
DmRP49 sense	GCTAAGCTGTCGCACAAATG
DmRP49 antisense	GAACTTCTTGAATCCGGTGGG

The three strains of flies used for tissue collection were raised in the same manner as the strains used for behavioral assays, isolated and then placed in the incubator until sexual maturation post-egression. Once the animals had matured, the males were placed on ice in order to anaesthetize them prior to removal of the abdomen. Abdomens were removed from ~50 male flies, and the remaining head, thorax, legs, and wings were placed in a tube nestled in dry ice to immediately freeze the tissue and prevent any degradation. Once an adequate amount of tissue had been collected, the tube was stored at -70° degrees Celsius until RNA isolation.

In order to isolate RNA, a baked mortar and pestle were placed into a container of liquid nitrogen. Once the temperature had appropriately lowered, the frozen tissue was poured into the mortar and ground by the pestle into a powder. Once ground, 500 ul of Trizol, chemical solution to prevent enzymatic activity, per mg of tissue is added in 100 ul increments to the powder and ground together to make a homogenous frozen mixture. After half of the allocated Trizol had been added, the mortar and pestle were transferred

to a slide warmer at ~65° C until the mixture had thawed and become liquid. Remaining Trizol was used to rinse the remaining tissue off of the pestle and to further homogenize the solution. Once all of the Trizol had been used, the mixture was pipetted 100 ul at a time into a 1.25 mL microcentrifuge tube. This was placed into the centrifuge for 5 minutes at 12,000 RPM to remove particulate; supernatant containing genetic material was transferred into a clean microcentrifuge tube. At this point, 200 ul of Chloroform:isoamyl alcohol mix (24:1) was added for each 1 mL of Trizol used in order to separate the genomic material. Solution was vortexed for 15 seconds until a cloudy and pink, incubated at room temperature for 2 to 3 minutes, and centrifuged for 15 minutes at 12,000 RMP and 4° **C**. After centrifugation, aqueous phase was carefully pipetted into a new tube with extra precaution not to disturb the organic phase and interphase. To precipitate RNA, 500 uL of 70% isopropyl alcohol per 1mL of starting Trizol was added to the tube and mixed well. This solution was transferred to an RNeasy Mini spin column and centrifuged for 15 seconds at 8,000 RPM. Flow through was discarded, 350 uL Buffer RW1 was added to the column, and the column was spun down again for 15 seconds at 8,000 RPM. Flow through was once again discarded. 10 uL Qiagen DNase I was mixed with 70 uL Buffer RDD very carefully as DNAse I is especially sensitive to physical denaturation. This mix was added to the column, incubated for 30 minutes at room temperature, and then 350 more uL of Buffer RW1 was added to the column. Column was then incubated for 5 minutes at room temperature, spun down for 15 seconds at 8000 x g, and flow through was discarded. To wash the filter, 500 uL Buffer RPE was added to the column and spun down for 15 seconds at 8000 x g, flow through was discarded. This step was done twice to ensure that the RNA was sufficiently washed

and no other genetic material had stuck to the filter. The second RPE wash was spun for 1 minute, and then the column was placed in a fresh 2 mL collection tube. This was then spun down for 2 minutes at maximum speed to dry the column, which was then placed in a 1.5 mL eppindorf tube. 30 uL RNase- free water was pipetted directly on the membrane in order to release the RNA from the filter; the RNA dissolved into the water. The tube was incubated at room temperature for 15 minutes and then spun down for 1 minute at maximum speed to elute the RNA. RNA is stored at -70° **C.**

cDNA was next synthesized from RNA in order to run qPCR. To create component 1, up to 5 uL RNA was mixed with 1 uL dNTP mix (10 mM stock), 1 uL $Oligo(dT)_{12\text{-}18}$ (0.5 ug/ul stock), and DEPC treated water to bring the mixture to 10 ul. This solution was incubated for 5 minutes at 65° C and then incubated on ice for at least one minute. Component two was then made by mixing 2 ul 10X RT buffer with 4 ul $MgCl_2$ (25 mM stock), 2 ul DTT (0.1 mM stock), and 1 ul RNase OUT (RNase inhibitor), then incubating for 2 min at 42° **C.** 1 ul of Superscript III was added to component two, which was then mixed with component 1 in a PCR tube and placed in the thermocycler for 50 minutes at 50° **C** and then terminated at 85° **C** for 5 minutes. cDNA could then be stored at -20° **C**.

In order to run the cDNA through quantitative real time PCR, 2 ul of cDNA was mixed with 4 ul of H_2O, 10 ul of Sybr green (a fluorescent marker), and 2 ul each of sense and antisense primer for a total of 20 ul per well. These mixtures pipetted directly into the plate that was placed into the CFX 960 for 40 cycles. The first step of the cycle, only completed once, was a 95° **C** hot start in order to efficiently denature the cDNA. Afterwards, the 40 cycles progressed through three steps of 95° **C** for 10 seconds, 55° **C**

for 10 seconds, and 72° **C** for 30 seconds to denature, anneal, and extend as with standard PCR protocol. The cDNA collected from each genotype, W1118, UAS:RNAi/UAS:RNAi, and SNMP-2:GAL4/UAS:RNAi, were amplified with each set of primers, RP49, SNMP-1, and SNMP-2, in triplicates, giving a total of 27 wells run through the thermocycler at a time. This was done two times in order to return 6 samples of each combination of cDNA and primers. Once the reaction had come to completion, samples were removed and the data was organized by Biorad CFX manager software.

2.5 Statistical analysis on qPCR

When quantification cycles of the samples had been collected, statistical analysis was done on these numbers in order to normalize the data and determine the difference in cycle numbers between samples. This data represents the difference in quantity of transcript being produced. To normalize the data, quantification cycle numbers for each of the 27 samples were organized into a spreadsheet. The mean of quantification cycles to cross threshold (cq) for RP49, ribosomal mRNA used as a control, was calculated for each genotype, yielding an average RP49 for each W1118, UAS:RNAi/UAS:RNAi, and SNMP-2:Gal4/UAS:RNAi. This mean was subtracted from each individual SNMP-1 and SNMP-2 cq of the corresponding genotype as a reference point. Once all of these numbers had been calculated, there were six sets of data with six data points, representing the six wells run for each sample. The six data sets were of the remaining primers, SNMP-1 and SNMP-2, for each genotype. Means for each of these data sets were calculated; in order to normalize these numbers and determine how many cycles occurred between each sample as they reached threshold, W1118 SNMP-1 and SNMP-2 means

were set to one. Adequate adjustments were made for corresponding samples, allowing the data to be viewed solely by the number of cycles that UAS:RNAi/UAS:RNAi SNMP-1 and SNMP-2 and SNMP-2:Gal4/UAS:RNAi SNMP-1 and SNMP-2 differed from W1118 SNMP-1 and SNMP-2 and from each other. The efficiency of the knockdown was calculated after Liu and Saint, 2002.

Chapter 3

Results

3.1 Behavioral Results - Female

Time spent in display of natural courtship behavior was recorded for each pair of flies within their chamber in order to determine the effect of the inhibition of SNMP-2 protein production. Expected behavior for male/female courtship is a high response, therefore, pairs exhibiting no courtship, or zeros, were not considered in statistical analysis. It seems that [SNMP-2:Gal4/UAS:RNAi] males show approximately the same preference towards target females as that of [W1118] males, 63% of time spent, which eliminates the possibility of this mating abnormality resulting from a generally elevated predisposition to attempting copulation. For 75% of the ten minutes in the chamber, [UAS:RNAi/UAS:RNAi] males mated with the target females.

Table 3.1

Percentage of Time in Courtship: Data sets of behavioral assays. N refers to number of pairs recorded. Mean of percentage of time spent in courtship.

Without zeros (data used for male/female)	S2:GAL4/ UAS:S2 v CSfemale	S2:GAL4/ UAS:S2 v CSmale	UAS:S2 v CSfemale	UAS:S2 v CSmale	W1118 v CSfemale	W1118 v CSmale
n	21	26	8	30	9	14
mean	0.63	0.54	0.75	0.44	0.63	0.28
median	0.68	0.51	0.79	0.43	0.65	0.33
var	0.052	0.064	0.028	0.044	0.082	0.032

The Wilcoxon Mann-Whitney test was done on the non-parametric data to determine if data sets were significantly different from one another. For sets with target

females, the test showed that data from [SNMP-2:Gal4/UAS:RNAi v CS f] and [W1118 v CS f] had a p-value of 0.89, data from [UAS:RNAi/UAS:RNAi v CS f] and [W1118 v CS f] had a p-value of 0.48, and data from [SNMP-2:Gal4/UAS:RNAi v CS f] and [UAS:RNAi/UAS:RNAi v CS f] had a p-value of 0.22.

Table 3.2

Wilcoxon P-Values (Mann-Whitney, non-parametric) comparing data sets of time spent in courtship. P-values less than 0.05 show a statistically significant difference; P-values less than 0.01 have a higher significance level. Data without zeros used to calculate male/female courtship; data with zeros used to calculate male/male courtship.

Without zeros	[S2:Gal4/UAS:RNAi] v CS male	[UAS:RNAi/UAS:RNAi] v CS female	[UAS:RNAi/UAS:RNAi] v CS male	[W1118] v CS female	[W1118] v CS male
[S2:Gal4/UAS:RNAi] v CS female	0.2523	0.2183	0.004755	0.894	0.0002421
[S2:Gal4/UAS:RNAi] v CS male		0.02691	0.1307	0.3551	0.002298
[UAS:RNAi/UAS:RNAi] v CS female			0.001193	0.4807	4.38E-05
[UAS:RNAi/UAS:RNAi] v CS male				0.07452	0.02491
[W1118] v CS female					0.003417

3.2 Behavioral Results – Male

Expected behavior for male/male courtship is a low response; therefore, non-responding pairs were considered significant and included in statistical analysis. [S2:Gal4/UAS:RNAi] males reported 12 non-respondents when mating male targets, [UAS:RNAi/UAS:RNAi] males had 5 instances of no response towards males, and [W1118] males did not respond in 18 cases when courting males. This data alone shows the significant difference between responses of experimental and wild-type strains to target animals. 56% of W1118 males did not engage in any courtship activity with target males while only 32% of [S2:Gal4/UAS:RNAi] males and 14% of

[UAS:RNAi/UAS:RNAi] males showed no courtship responses to target males. The [SNMP-2:Gal4/UAS:RNAi] males spent an average of 37% of time in the chamber courting CS males, which is much greater than the 12% of time that [W1118] males spent mating with CS males. [UAS:RNAi/UAS:RNAi] males collectively spent 44% of their time engaging in courtship behaviors with target males.

Table 3.3

Data sets and Non-respondents: Number of data sets (n), number of males showing no response to target (#zeros), percentage of males showing no response to target (%zeros).

	[S2:GAL4/ UAS:RNAi] v CS female	[S2:GAL4/ UAS:RNAi] v CS male	[UAS:RNAi/ UAS/RNAi] v CS female	[UAS:RNAi/ UAS/RNAi] v CS male	[W1118] v CS female	[W1118] v CS male
n	36	38	11	35	15	32
# zeros	15	12	3	5	6	18
% zeros	42%	32%	27%	14%	40%	56%

Table 3.4

Percentage of Time in Courtship: Data sets of behavioral assays. N refers to number of pairs recorded. Mean of percentage of time spent in courtship.

With zeros (data used for male/male)	S2:GAL4/ UAS:S2 v CSfemale	S2:GAL4/ UAS:S2 v CSmale	UAS:S2 v CSfemale	UAS:S2 v CSmale	W1118 v CSfemale	W1118 v CSmale
n	36	38	11	35	15	32
mean	0.37	0.37	0.55	0.38	0.38	0.12
median	0.32	0.39	0.72	0.39	0.25	0.00
var	0.129	0.109	0.144	0.062	0.150	0.034

The Mann-Whitney test was also done for the male/male pairs; data from [SNMP-2:Gal4/UAS:RNAi v CS m] and [W1118 v CS m] had a significant p-value of approximately 0.0017, whereas [UAS:RNAi/UAS:RNAi v CS m] and [W1118 v CS m] had a p-value of $3.299e^{-5}$. The p-value of data sets [SNMP-2:Gal4/UAS:RNAi v CS m] and [UAS:RNAi/UAS:RNAi v CS m] was about 0.81.

Table 3.5

Wilcoxon P-Values (Mann-Whitney, non-parametric) comparing data sets of time spent in courtship. P-values less than 0.05 show a statistically significant difference; P-values less than 0.01 have a higher significance level. Data without zeros used to calculate male/female courtship; data with zeros used to calculate male/male courtship.

With zeros	[S2:Gal4/UAS:RNAi] v CS male	[UAS:RNAi/UAS:RNAi] v CS female	[UAS:RNAi/UAS:RNAi] v CS male	[W1118] v CS female	[W1118] v CS male
[S2:Gal4/UAS:RNAi] v CS female	0.8723	0.1626	0.7801	0.8723	0.01175
[S2:Gal4/UAS:RNAi] v CS male		0.1226	0.8069	0.992	0.001563
[UAS:RNAi/UAS:RNAi] v CS female			0.0908	0.3401	0.00218
[UAS:RNAi/UAS:RNAi] v CS male				0.782	3.30E-05
[W1118] v CS female					0.0407

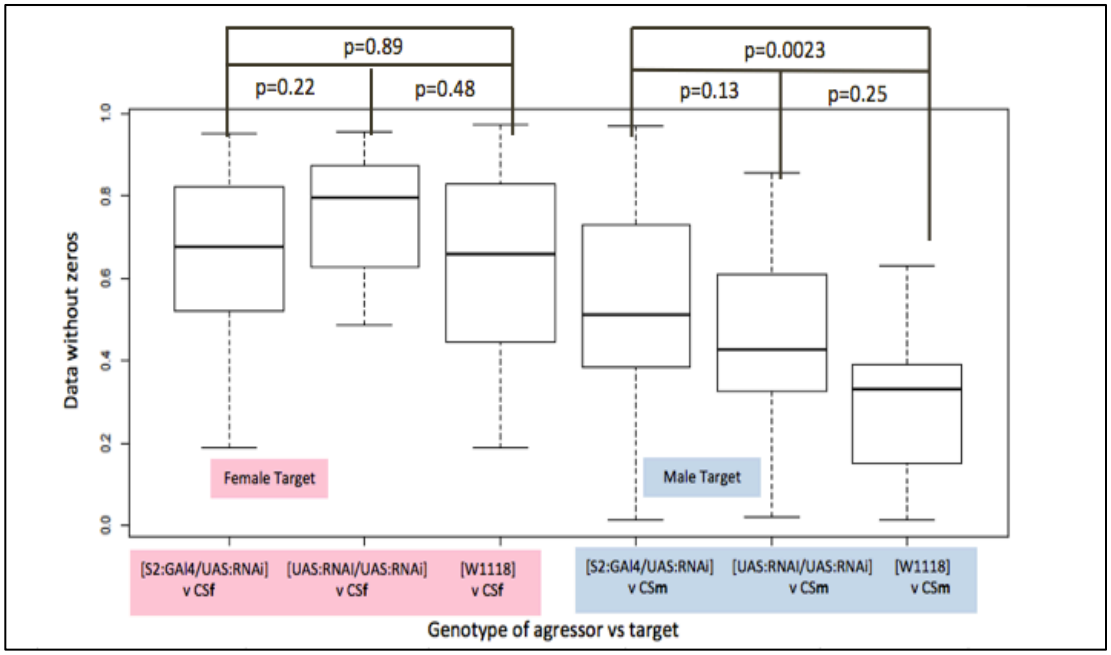

Figure 3.1

Whisker plot showing non-parametric assessment of time spent courting target. Left hand side shows preference toward female target, right hand side shows preference toward male target. X-axis shows genotype with target and Y-axis shows percentage of time out of 100 spent in courtship. P-values on top show significance of statistical difference between data sets. Whiskers indicate maximum and minimum data points, while box hinges define the interquartile range. Horizontal line indicates median.

3.3 Quantitative Real Time PCR Results

Means of cq calculated for samples run with RP49 primer were 26.13 for [W1118], 22.35 for [UAS:RNAi/UAS:RNAi], and 24.465 for [S2:Gal4/UAS:RNAi]. The reporter line, followed by the knockdown, showed the highest expression of RP49; lowest expression of RP49 was shown by W1118.

Table 3.6

Cycle Numbers of Individual Samples: Table of each quantification cycles reaching threshold wavelength for each set of primers used and each genotype of cDNA used for both plates run in the thermocycler.

RP49	First Run			RP49	Second Run		
cDNA	Cycle 1	Cycle 2	Cycle 3	cDNA	Cycle 1	Cycle 2	Cycle 3
W1118	26.42	26.22	26.15	W1118	26.05	26.02	25.92
GD	22.22	22.29	22.21	GD	22.53	22.45	22.42
RNAi	24.3	24.27	24.54	RNAi	24.55	24.5	24.63
SNMP-1				SNMP-1			
cDNA	Cycle 1	Cycle 2	Cycle 3	cDNA	Cycle 1	Cycle 2	Cycle 3
W1118	24.71	24.64	24.96	W1118	24.89	24.85	25
GD	21.41	21.27	21.26	GD	21.55	21.27	21.35
RNAi	23	23.04	23.06	RNAi	23.13	23.07	22.92
SNMP-2				SNMP-2			
cDNA	Cycle 1	Cycle 2	Cycle 3	cDNA	Cycle 1	Cycle 2	Cycle 3
W1118	23.59	23.37	23.67	W1118	23.48	23.51	23.59
GD	23.69	23.44	24.17	GD	24.3	24.15	24.18
RNAi	25.27	25.4	25.22	RNAi	25.58	25.3	25.28

Table 3.7

Cycle Numbers of Individual Samples with RP49 Mean for Each Genotype: Pooled data from Table 3.6 organized by genotype. Means for RP49 primers for each genotype calculated to be used in statistical analysis.

Genotype	Primers	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean
W1118	RP49	26.42	26.22	26.15	26.05	26.02	25.92	26.13
	S1	24.71	24.64	24.96	24.89	24.85	25	
	S2	23.59	23.37	23.67	23.48	23.51	23.59	
GD	RP49	22.22	22.29	22.21	22.53	22.45	22.42	22.35
	S1	21.41	21.27	21.26	21.55	21.27	21.35	
	S2	23.69	23.44	24.17	24.3	24.15	24.18	
RNAi	RP49	24.3	24.27	24.54	24.55	24.5	24.63	24.465
	S1	23	23.04	23.06	23.13	23.07	22.92	
	S2	25.27	25.4	25.22	25.58	25.3	25.28	

After normalizing the data for the purpose of statistical analysis, it can be determined that the concentration of SNMP-1 reaches threshold wavelength 0.29 cycles after W1118 in the reporter line and 0.14 cycles before W1118 in the knockdown.

Therefore, the amount of SNMP-1 protein being synthesized is relatively consistent in all three genotypes.

Table 3.8

Differences in Cycle Number with Respect to RP49 Mean: Normalizing data of quantification cycles by subtracting RP49 mean (for each respective genotype) from individual sample numbers. Normalized means show relative cycle differences between genotypes for SNMP-1 and SNMP-2.

Genotype	Primers	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	Normalized
W1118	S1	-1.42	-1.49	-1.17	-1.24	-1.28	-1.13	-1.29	1.00
GD	S1	-0.94	-1.08	-1.09	-0.8	-1.08	-1	-1.00	1.29
RNAi	S1	-1.46	-1.42	-1.4	-1.33	-1.39	-1.54	-1.42	0.86
W1118	S2	-2.54	-2.76	-2.46	-2.65	-2.62	-2.54	-2.60	1.00
GD	S2	1.34	1.09	1.82	1.95	1.8	1.83	1.64	5.23
RNAi	S2	0.81	0.94	0.76	1.12	0.84	0.82	0.88	4.48

The concentration of SNMP-2 protein reaches threshold 4.23 cycles after W1118 in the reporter line and 3.48 cycles after W1118 in the knockdown. Cycle numbers at which SNMP-1 and SNMP-2 transcripts cross threshold wavelength are graphically represented according to genotype, with W1118 set to 1 as a relative point of comparison. P-values calculated for these data sets were $1.256e^{-07}$ for W1118 and the reporter line, $6.189e^{-13}$ for W1118 and the knockdown, and 0.0017 for the reporter and knockdown lines. All of these values are statistically significant, but the p-values from examining data sets W1118/reporter and W1118/knockdown show the strongest significance.

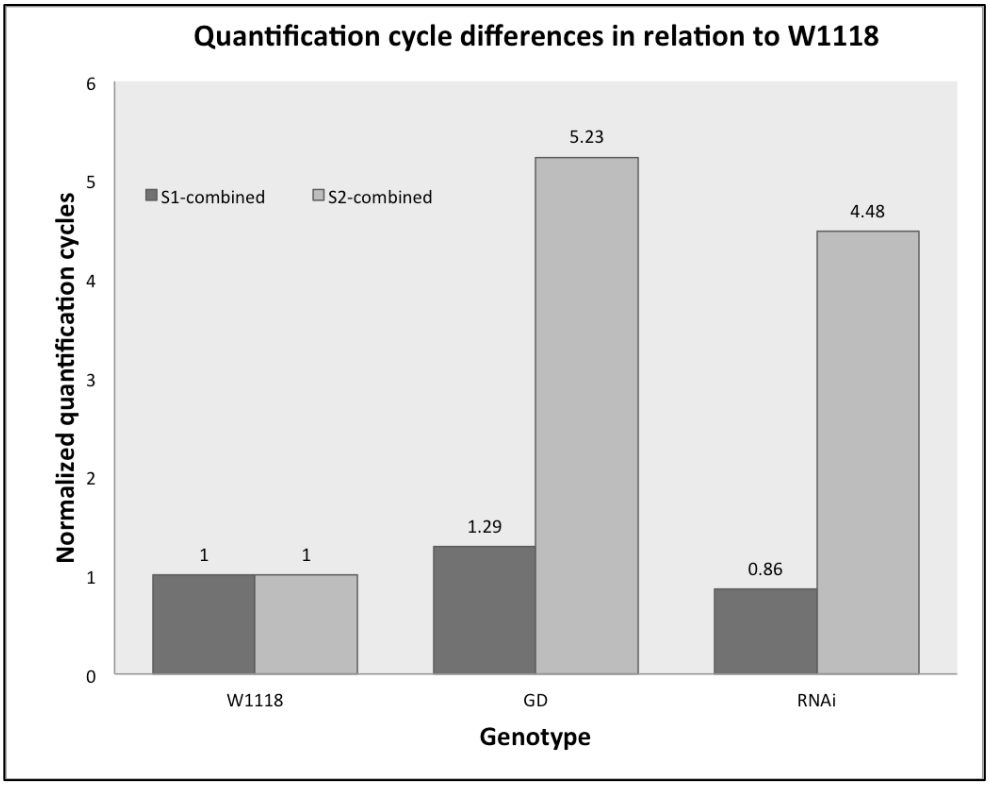

Figure 3.2

Graphical representation in the differences between normalized data values for quantification cycles reaching threshold in SNMP-1 and SNMP-2. For SNMP-1, the reporter line reaches threshold 1.29 cycles after W1118 and the knockdown line reaches threshold 0.14 cycles before W1118. For SNMP-2, the reporter line reaches threshold 5.23 cycles after W1118 and the knockdown line reaches threshold 4.48 cycles after W1118.

Table 3.9

P-values were calculated to measure the difference in between each data set. For W1118 and the reporter, p-values were 0.0031 for SNMP-1 and $1.26e^{-07}$ for SNMP-2. For W1118 and the knockdown, p-values were 0.0645 for SNMP-1 and $6.19e^{-13}$ for SNMP-2. For the reporter and the knockdown, p-values were $3.77e^{-05}$ for SNMP-1 and 0.0018 for SNMP-2.

P-values	S1	S2
W1118-GD	0.00310884	1.25671E-07
W1118-RNAi	0.06453476	6.18968E-13
GD-RNAi	3.7714E-05	0.001794616

The efficiency of the knockdown was calculated after Liu and Saint, 2002. Amplification curves show that mRNA transcript is doubled after two cycles, and the amplification efficiency is around 41%. RNAi efficiency was calculated as 50%, using the comparative C_T (threshold cycle number) method to assess levels of relative gene expression.

Amplification Efficiency	Relative Gene Expression
$E = (R_NA/R_NB)^{1/CtA-CtB} -1$	$R_{N,b}/R_{N,a}=(1+E)^{-\Delta\Delta CT}$
$E = (500/1{,}000)^{1/26-28} -1$	$R_{N,b}/R_{N,a} = (1.4142)^{-2}$
$E = (.5)^{-1/2}-1$	$R_{N,b}/R_{N,a=}$ 0.50
E= 0.4142	50% Knockdown

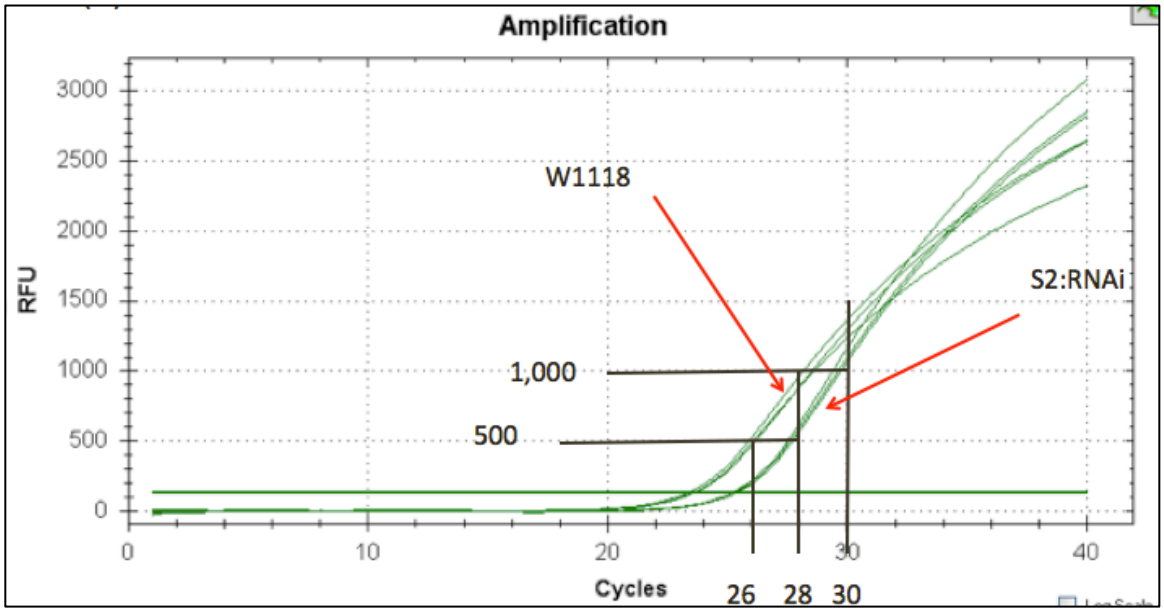

Figure 3.3

Amplification Efficiency and Relative Gene Expression for the amplification of SNMP-2 mRNA transcript in two genotypes, [W1118] and [SNMP-2:Gal4/RNAi].

Chapter 4

Discussion

Given the understanding that an animal's loss of a particular region, appendage, organ, neuron, or receptor can significantly hinder natural behavior, it is acceptable to assume that decreasing the production of one distinct protein can also cause deviations from expected social or physical phenotypes. The effect of removal or reduction of sensory neuron membrane protein 2 is one example of the impact that down-regulating a protein can have on an organism. Although SNMP-2 is not thought to behave as a receptor, it is a membrane bound protein essential for standard function in *Drosophila.*

Under normal circumstances (W1118), males will display courtship behavior towards females 63% of the time and towards males only 12% of the time; this shows a 51% difference between male/female and male/male courtship. Considering the genotype [SNMP-2:Gal4/UAS:RNAi], males spend 63% of their time courting females and 37% of their time courting males, closing the gap to only 26% difference in preference of female over male. After decreasing the production of SNMP-2 protein in wild type males to get the knockdown, we observe no difference in male/female courtship. However, knockdown males exhibit a 25% increase in tendency to court other males from that of W1118. The p-value of data sets between wild type and knockdown males courting females is 0.87, which does not indicate a significant statistical difference. The preference of males from these two genotypes is essentially equivalent. On the other

hand, the p-value of data sets between these two genotypes courting males is 0.0016. This value is well under 0.01, which shows a particularly significant statistical difference. The 25% increase in the tendency to initiate the courtship sequence is suggests a noteworthy distinction between the behaviors of the two genotypes.

We know that the behavior of these animals was affected only by the reduction of SNMP-2 transcripts by looking at qPCR data. When amplifying SNMP-1 in all three genotypes, the samples reached threshold wavelength at essentially the same time, indicating that all three genotypes were synthesizing similar amounts of SNMP-1 transcript. With regards to samples including SNMP-1 primers, the p-value comparing W1118 and the knockdown was 0.06, a number indicating that the difference between data sets is insignificant. Had there been a difference between the quantification cycles of SNMP-1 products, that difference would suggest that the gene expression of SNMP-1 had been reduced and subsequently could potentially have an effect on the behavior of the animal. Since the amount of SNMP-1 transcript synthesized by all genotypes was essentially the same, we can assume that the SNMP-1 protein was equally expressed in each of the genotypes.

When amplifying SNMP-2, the samples containing knockdown cDNA reach threshold wavelength 3.48 cycles after the samples containing wild type cDNA. The p-value comparing these sets of data returned a significant value of $6.19e^{-13}$, which shows that there is a sizeable difference between the data sets. The large interval in between the cq's of these two samples represents the difference in the amount of mRNA being transcribed. The 3.48 additional cycles it takes for the knockdown to synthesize mRNA

transcript indicates about a 45% reduction in protein production, meaning that the RNAi was successful in reducing SNMP-2 expression.

Given this data, it seems that there could be a correlation between the down regulation of SNMP-2 and the inclination of males to engage in courtship with other males. One question that arises is the role that SNMP-2 itself plays in the sensory process. Experiments were done in the dark, so it is safe to assume that this protein contributes to perception of chemosensory molecules, either gustatory or olfactory. Morphological data indicates that SNMP-2 is expressed in olfactory sensory neurons, but is primarily expressed in gustatory sensory neurons containing gustatory receptors (Sparks). Since the reduction of the protein has no effect on male/female interactions, we can infer that SNMP-2 only relates to perception of chemosensory molecules secreted from male *Drosophila.* It is possible that this protein is involved in inhibiting the act of males mating with other males. The down regulation of SNMP-2 may decrease the sensitivity towards chemosensory molecules secreted by males, thereby decreasing the inhibitory response normally associated with that sensory perception.

Although we do see a significant difference between wild type and knockdown males in the percentage of time spent courting other males, we also observed a slightly higher increase in the tendency of males from the reporter line to court other males. Knockdown males spent 37% of the time courting other males while the males from the reporter line spent 38% of their time courting other males. P-values showed that the statistical difference between reporter and wild type, $3.30e^{-05}$, was very significant; statistical difference between reporter and knockdown, 0.81, was not significant. This

shows that the reporter and knockdown lines exhibited fundamentally consistent behavior, which was much greater than that of the wild type line.

Male-female courtship in the reporter line was also amplified; males from the reporter line spent 75% of their time in courtship with females whereas males from wild type and knockdown lines only spent 63% in courtship. P-values showed that the statistical difference between reporter and wild type, 0.48, was not significant, and neither was the statistical difference between reporter and knockdown, 0.21. Therefore, although the likelihood of male-female courtship behavior in the reporter line is elevated, it is insignificant.

Considering the qPCR data, results comparing the amounts of mRNA transcripts synthesized were consistent with behavioral data. It seems that similar amounts of SNMP-2 mRNA present in the knockdown and reporter are very similar, and much higher than that of the wild type. The samples from the reporter line produce a sufficient amount of mRNA transcript to reach the set arbitrary threshold 4.23 cycles after wild-type, and the knockdown line produces enough to cross threshold 3.48 cycles after wild-type. This data shows that the reporter line is synthesizing enough mRNA transcript to cross threshold 0.75 cycles before that of the knockdown line. P-values indicate that the difference between wild type and reporter line is statistically significant at $1.26e^{-07}$, as well as the difference between wild type and knockdown line at $6.19e^{-13}$. The difference between knockdown and reporter lines is less statistically significant according to the p-value of 0.0018.

The reduced production of SNMP-2 protein seems to have a direct correlation with the increased affinity of males to mate with other males. However, functionally the

reporter line [UAS:RNAi/UAS:RNAi] should not be reducing the amount of SNMP-2 mRNA transcript without the Gal4 protein binding to UAS to activate transcription of hairpin RNAs. The knockdown line contains the SNMP-2:Gal4 construct which activates UAS and drives expression of hairpin RNAs. Clearly the UAS:RNAi is activating gene transcription without activator protein Gal4, which means the sequence is leaking and silencing SNMP-2 without Gal4. In both cases of the reporter and knockdown lines, SNMP-2 reduction is evident. This may be due to the fact that the reporter line is homozygous and contains two copies of the inducible UAS:RNAi construct, whereas the knockdown line only contains one copy. It seems that this excess of UAS:RNAi construct is driving expression of hairpin RNA and subsequent degradation of SNMP-2 mRNA in the reporter line, even without the presence of Gal4 activator protein. Future experiments could include crossing the reporter line with W1118 to create a heterozygous genotype for the UAS:RNAi construct as a control, so that the control and experimental lines contained equivalent amounts of UAS:RNAi. Another solution to increase the efficacy of RNAi gene targeting could be to introduce additional dicer enzyme to cleave the double stranded RNAs into siRNAs and improve the ability degrade mRNA.

These behavioral studies show that the reduction of the synthesis of one particular protein can significantly influence expected behavior. This is biologically relevant because we can see the consequences that targeting the expression of a singular gene can have on phenotypic outcomes. This knowledge can be adapted to genetics in humans, perhaps to include gene therapy in order to down regulate or up regulate synthesis of particular proteins responsible for disease. *Drosophila* genetic research is an irreplaceable tool when it comes to discoveries connected to gene manipulation and phenotypic effects.

Hopefully our understandings of molecular processes will develop sufficient techniques to be able to target illnesses and generate adequate treatments and cures to eradicate maladies that continue to plague humans and destroy lives.

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